Recombinant Gene Expression and ^{1}H NMR Characteristics of the Kringle (2 + 3) Supermodule: Spectroscopic/Functional Individuality of Plasminogen Kringle Domains†

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ABSTRACT: The plasminogen kringle 2 ($K2_{HPg}$) and kringle 3 ($K3_{HPg}$) modules occur in tandem within the polypeptide segment that affords the heavy chain of plasmin. The $K2_{HPg}$ and $K3_{HPg}$ are unique among the plasminogen kringle domains in that they also are linked to each other via the Cys^{169}−Cys^{297} (Cys^4 of $K2_{HPg}$ to Cys^{43} of $K3_{HPg}$, kringle numbering convention) disulfide bridge, thus generating a $K2_{HPg}$-$K3_{HPg}$ "supermodule". The kringle (2 + 3) sequence of human plasminogen (r-EE[$K2_{HPg}K3_{HPg}$]DS) was expressed in *Escherichia coli*, using an expression vector containing the phage T5 promoter/operator N250PSN250P29 and the codons for an N-terminal hexahistidine tag to ensure the isolation of the recombinant protein by affinity chromatography on Ni^{2+}−nitrilotriacetic acid/agarose under denaturing and reducing conditions. Kringle (2 + 3) was refolded in the presence of glutathione redox buffer. By taking advantage of the lysine affinity of kringle 2, the protein was purified by affinity chromatography on lysine-Bio-Gel. Recombinant kringle (2 + 3) was identified by amino acid composition, N-terminal sequence and mass determination. The ^{1}H NMR spectrum shows that the intact r-$K2_{HPg}K3_{HPg}$ is properly folded. By reference to spectra of the individual kringles, r-$K2_{HPg}$ and r-$K3_{HPg}$, resonances of the $K2_{HPg}$ and $K3_{HPg}$ components in the spectrum of the intact r-$K2_{HPg}K3_{HPg}$ can be readily distinguished. The strictly conserved Leu^{46} residue (kringle residue numbering convention) yields δ-methyl signals that are characteristic for $K2_{HPg}$ and $K3_{HPg}$, exhibiting chemical shifts of −0.87 and −0.94 ppm, respectively, which are distinct from those of $K1_{HPg}$, $K4_{HPg}$, and $K5_{HPg}$, (−1.04 to −1.05 ppm). Thus, the high-field Leu^{46} signals from $K2_{HPg}$ and $K3_{HPg}$ are well resolved from those of other kringles and can be identified unambiguously in spectra of the $K1_{HPg}K2_{HPg}K3_{HPg}$ elastolytic fragment of plasminogen as well as in spectra of Glu−plasminogen. Overall, r-$K2_{HPg}K3_{HPg}$ exhibits broader resonance line widths than does the $K1_{HPg}$ component, consistent with a lesser mobility of the $K2_{HPg}K3_{HPg}$ segment within the $K1_{HPg}K2_{HPg}K3_{HPg}$ fragment, a reflection of the extra structural constraint imposed by the disulfide bridge linking $K2_{HPg}$ to $K3_{HPg}$. The ligand 6-aminohexanoic acid (6-AHA), which is known to interact with r-$K2_{HPg}$ but not with r-$K3_{HPg}$, selectively perturbs K2 aromatic signals in the intact r-$K2_{HPg}K3_{HPg}$ spectrum while leaving K3 resonances largely unaffected. Association constant (K_a) values for 6-AHA determined from ^{1}H NMR ligand titration experiments yield $K_a \approx 2.2 \pm 0.3$ mM^{-1} for the intact r-$K2_{HPg}K3_{HPg}$, comparable to $K_a \approx 2.3 \pm 0.2$ mM^{-1} determined for the isolated r-$K2_{HPg}$, which demonstrates that the interactions of 6-AHA with the $K2_{HPg}$ ligand-binding site are not significantly affected by the neighboring $K3_{HPg}$ domain within the intact r-$K2_{HPg}K3_{HPg}$ supermodule.

The polypeptide units of individual kringle domains consist of about 80 amino acid residues each, with a characteristic 1−6, 2−4, 3−5 Cys−Cys bridge pattern. In plasminogen (Pg),[1] the segment that is to become the heavy or A-chain of plasmin upon activation contains five kringles, some of which are known to interact with lysyl side chains, particularly those exposed by the fibrin matrix of blood clots. Among the heavy chain domains, the sequential kringles 2 and 3 are peculiar in that they occur cross-linked via a disulfide bond that, in the human Pg (HPg), bridges Cys^{169} in kringle 2 ($K2_{HPg}$) to Cys^{297} in $K3_{HPg}$ (Figure 1).[2] ^{1}H NMR solution structures of $K1_{HPg}$ (Rejante & Llinás, 1994), $K4_{HPg}$ (Atkinson & Williams, 1990), $K4_{EPg}$ (Cox *et al.*, 1994), urokinase kringle (K_{uPA}) (Li *et al.*, 1994; Hansen *et al.*, 1994), and tissue-type plasminogen activator K2 ($K2_{tPA}$) (Byeon & Llinás, 1991) have been reported. Crystallographic structures of the prothrombin kringle 1 ($K1_{PT}$) (Tulinsky *et al.*, 1988a) and $K2_{PT}$ (Arni *et al.*, 1993) as well as $K1_{HPg}$ (Wu *et al.*, 1994), $K4_{HPg}$ (Mulichak *et al.*, 1991; Wu *et al.*, 1991), and $K2_{tPA}$ (de Vos *et al.*, 1992) have been solved via X-ray diffraction methods.

Lysine and zwitterionic analogs, such as 6-aminohexanoic acid (6-AHA), are ligands for $K1_{HPg}$, $K4_{HPg}$, and $K5_{HPg}$ as well as for the $K2_{tPA}$ (Winn *et al.*, 1980; De Marco *et al.*, 1987; Ramesh *et al.*, 1987; Thewes *et al.*, 1990; Byeon *et al.*, 1995). Except for minor details, the reported crystal and solution structures of the various kringles support the original

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Chart 1: Sequence Alignment of the Human Plasminogen Kringles

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          1        10        20        30        40        50        60        70        80
          |        |         |         |         |         |         |         |         |
K1 CKTGNGKNYRGTMSKTKNGITCQKWSSTSPHRP-RFSPATHPSEGLEENYCRNPDNDPQGPWCYTTDPEKRYDYCDILEC
K2 CMHCSGENYDGKISKTMSGLECQAWDSQSPHAHG-YIPSKFPNKNLKKNYCRNPDREL-RPWCFTTDPNKRWELCDIPRC
K3 CLKGTGENYRGNVAVTVSGHTCQHWSAQTPHTHNR-TPENFPCKNLDENYCRNPDGKR-APWCHTTNSQVRWEYCKIPSC
K4 CYHGDGQSYRGTSSTTTTGKKCQSWSSMTPHRHQK-TPENYPNAGLTMNYCRNPDADK-GPWCFTTDPSVRWEYCNLKKC
K5 CMFGNGKGYRGKRATTVTGTPCQDWAAQEPHRHSIFTPETNPRAGLEKNYCRNPDGDVGGPWCYTTNPRKLYDYCDVPQC
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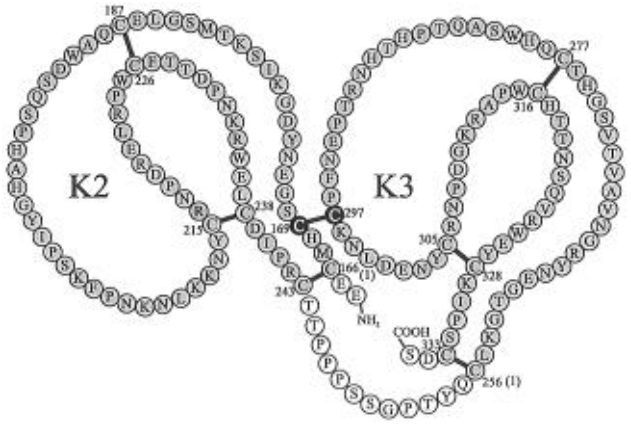

FIGURE 1: Primary structure of the human plasminogen kringle (2 + 3) supermodule. Amino acids residues are labeled according to the standard one-letter code and sequences corresponding to the individual kringle 2 (K2) and kringle 3 (K3) domains are highlighted by a darkened background. First residues of the individual domains' sequences, Cys^{166} (K2) and Cys^{256} (K3), are indicated by "(1)". Cystine bridges are denoted by solid bars; residues that structure the unique interkringle $Cys^{169}-Cys^{297}$ link are marked by reverse, white-on-black, labels.

model of the ligand binding site (Tulinsky *et al.*, 1988b) which comprises the aromatic residues (kringle residue numbering convention, Chart 1)[2] Phe^{36}, Trp^{62}, Tyr^{64}, Tyr^{72}, and Tyr^{74} in $K1_{HPg}$ (Rejante & Llinás, 1994), Trp^{62}, Phe^{64}, Trp^{72}, and Tyr^{74} in $K4_{HPg}$ (Ramesh *et al*., 1987; Tulinsky *et al.*, 1988a; Atkinson & Williams, 1990; Wu *et al.*, 1991), and Tyr^{36}, Trp^{62}, His^{64}, Trp^{72}, and Tyr^{74} in $K2_{tPA}$ (Byeon & Llinás, 1991; de Vos *et al.*, 1992; Byeon *et al.*, 1995). As indicated by these studies, the ϵ-amino group of the zwitterionic ligand 6-AHA ion pairs with the acidic side chains of Asp^{55} and Asp^{57} in both $K2_{tPA}$ and $K4_{HPg}$, while the ligand carboxylate group interacts with basic polar groups, variously provided by the $Lys^{34,35}$, Arg^{69}, and/or Arg^{71} side chains, situated within the constellation of residues that surrounds the binding site.

Recently, each of the individual recombinant HPg kringles have been obtained via expression in *Escherichia coli* (Menhart *et al.*, 1991; 1993; Nielsen *et al.*, 1993; Marti *et al.*, 1994). This includes the recombinant gene expression of a tandem $K4_{HPg}K5_{HPg}$ construct (Menhart *et al.*, 1993). In this paper, we present the expression in *E. coli*, purification, chemical identification, folding, and disulfide bridge formation of r-$K2_{HPg}K3_{HPg}$. The ^{1}H NMR spectra show that the obtained protein exhibits proper "kringle" folding. The study demonstrates that ^{1}H NMR spectroscopy can distinguish individual domains and their binding site components within the r-$K2_{HPg}K3_{HPg}$ "supermodule". The spectral individuality of the K2 and K3 domains is also apparent for the $K1_{HPg}K2_{HPg}K3_{HPg}$ fragment as well as for intact Glu−plasminogen. ^{1}H NMR also enables the ligand binding profile of the K2 unit within r-$K2_{HPg}K3_{HPg}$ to be monitored.

MATERIALS AND METHODS

Biochemical Procedures. Proteins. Polyclonal goat anti-HPg sera and alkaline phosphatase conjugated to rabbit antibodies against goat IgG were purchased from Sigma. Klenow fragment of DNA polymerase I, calf intestinal alkaline phosphatase, and restriction endonucleases were obtained from Boehringer Mannheim. *Taq* DNA polymerase, T4 polynucleotide kinase, and T4 DNA ligase were purchased from Promega.

Media for Chromatography. Lysine-Bio-Gel P-300 (Bio Rad) was prepared according to Brunisholz *et al.* (1979). Ni^{2+}−NTA/agarose was purchased from Qiagen. Agarose and Prep-A-Gene DNA purification matrix were obtained from Bio-Rad. Sephadex G-50 f was purchased from Pharmacia.

Bacterial Strains and Plasmids. E. coli strain M15 (F^- Str^r *lac*Z^{del}) (Zamenhof & Villarejo, 1972) was purchased from Qiagen and used for expression of r-$K2_{HPg}K3_{HPg}$. This strain was grown on 2×YT medium, containing 100 mg of ampicillin/mL and 25 mg of kanamycin/mL ($2\times YT_{amp/kana}$ medium). *E. coli* strain HB101 was used for routine transformations and plasmid preparations. Plasmid pQE-8 was obtained from Qiagen. It carries the promoter/operator element N250PSN250P29, (H. Bujard and M. Lanzer, unpublished results), a synthetic ribosomal binding site RBSII, the transcriptional terminator t_0 of the phage λ, the promoter-free gene for chloramphenicol acetyltransferase with its genuine translational signals, the transcriptional

[1] Abbreviations: 1D, one-dimensional; 2D, two-dimensional; 6-AHA, 6-aminohexanoic acid; CM, carboxymethyl; COSY, two-dimensional chemical shift correlated spectroscopy; DTT, 1,4-dithio-*dl*-threitol; EPg, equine plasminogen; FXa, activated coagulation factor X; Glu−HPg, intact, Glu^1 N-terminus, HPg; HPg, human plasminogen; IPTG, isopropyl β-D-thiogalactopyranoside; $K1_{HPg}$, kringle 1 domain of HPg ($Cys^{84}-Cys^{162}$), generated as fragment $Tyr^{80}-Glu^{165}$ of HPg; $K2_{HPg}$, kringle 2 domain of HPg ($Cys^{166}-Cys^{243}$), generated as r-$K2_{HPg}$; $K3_{HPg}$, kringle 3 domain of HPg ($Cys^{256}-Cys^{333}$), generated as r-$K3_{HPg}$; $K4_{HPg}$, kringle 4 domain of HPg ($Cys^{358}-Cys^{435}$), generated as fragment $Val^{355}-Ala^{440}(Val^{442})$ of HPg; $K4_{EPg}$, kringle 4 domain of EPg ($Cys^{358}-Cys^{435}$), generated as fragment $Val^{355}-Ser^{441}$ of EPg; $K1_{HPg}K2_{HPg}K3_{HPg}$, kringle (1 + 2 + 3) segment of HPg, generated as fragment $Tyr^{80}-Val^{338}$ of HPg; K_a, ligand−kringle equilibrium association constant; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE-correlated spectroscopy; NTA, nitrilotriacetic acid; pH*, glass electrode pH reading, uncorrected for ^{2}H isotope effect; PCR, polymerase chain reaction; Pg, plasminogen; PT, prothrombin; r-$K2_{HPg}$, recombinant plasminogen kringle 2 with C169G mutation (r-C162T/E163S/EE[$K2_{HPg}$/C169G]T); r-$K3_{HPg}$, recombinant plasminogen kringle 3 with Cys^{297} blocked with glutathione (r-TYQ-[$K3_{HPg}$]DS; Cys^{297}−glutathione) and an attached N-terminal hexahistidine tag; r-$K2_{HPg}K3_{HPg}$, recombinant human plasminogen kringles (2 + 3) (r-EE[$K2_{HPg}K3_{HPg}$]DS) and an attached N-terminal hexahistine tag; RP-HPLC, reversed-phase HPLC; TFA, trifluoroacetic acid; TOCSY, two-dimensional total correlation spectroscopy; tPA, human tissue-type plasminogen activator; uPA, human kidney-type plasminogen activator (urokinase).

[2] According to the kringle residue numbering convention, which is based on homology alignment against the kringle 5 (Llinás *et al.*, 1983; Tulinsky *et al.*, 1988b), Pg residues Cys^{169} and Cys^{297} correspond to Cys^4 and Cys^{43} of $K2_{HPg}$ and $K3_{HPg}$, respectively.

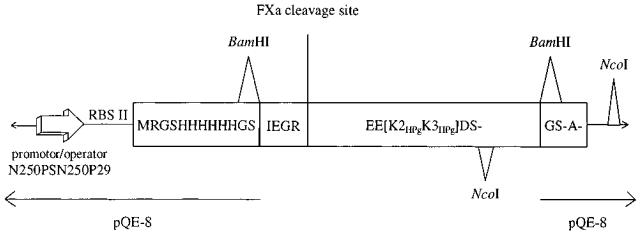

FIGURE 2: Construct of the expression vector for r-$K2_{HPg}K3_{HPg}$. The 5′-primer was used to introduce a *Bam*HI restriction endonuclease and a FXa recognition site I-E-G-R before the sequence of $K2_{HPg}$ (N-terminus: Glu^{164} of HPg) and the 3′-primer to introduce a stop codon (−) and a *Bam*HI restriction endonuclease site after the sequence of $K3_{HPg}$ (C-terminus: Ser^{335} of the HPg sequence). The digestion with *Nco*I of the expression vector containing the r-$K2_{HPg}K3_{HPg}$-coding construct generated a fragment of 745 bp.

terminator T1 of the *E. coli rrn*B operon, and a β-lactamase-selectable marker, and it encodes an N-terminal histidine tag. Plasmid pREP4, which expresses elevated levels of lac repressor and carries the gene for neomycin phosphotransferase, was also purchased from Qiagen. Plasmid pPLGKG contains the complete cDNA sequence of HPg and was kindly provided by Prof. L. O. Hedén (University of Lund, Sweden).

DNA Manipulations. Plasmid DNA was isolated according to the procedure of Birnboim and Doly (1979). Purification of large-scale plasmid preparations was performed on Qiagen tip-100 columns. Oligonucleotide primers were synthesized by Microsynth and finally purified on NAP-5 columns (Pharmacia). DNA sequences were determined by the alkaline denaturation method of double-stranded DNA (Tonnequzzo *et al.*, 1988) and sequenced by the dideoxynucleotide chain termination technique (Sanger *et al.*, 1977) with the Sequenase reagent kit from USB. The ethidium bromide stained cDNA fragments were excised from 0.8% or 2% agarose gels and extracted with the Prep-A-Gene DNA purification matrix.

Construction of the Expression Vector pqe-8$K2_{HPg}K3_{HPg}$. The cDNA segment of $K2_{HPg}K3_{HPg}$ was amplified from pPLGKG by PCR using the following two synthetic primers:

(1) K2 5′-primer, 5′-GCGGATCCATCGAGGGTAGA-GAGGAATGTATGCATTGCAGT-3′. This primer binds to the noncoding strand of the HPg cDNA. A *Bam*HI restriction endonuclease site and a cDNA segment that encodes for the FXa cleavage site were introduced upstream of the codon for Glu^{164}.

(2) K3 3′-primer, 3′-GGCAGGACACTGAGGATCCC-TAGGCG-5′. The 3′-primer, complementary to a region of the coding strand, was used to introduce a stop codon and a *Bam*HI restriction endonuclease site after the codon for Ser^{335}. The amplified $K2_{HPg}K3_{HPg}$ cDNA was cleaved with *Bam*HI, purified on a 2% agarose gel, and cloned into *Bam*HI-cleaved and dephosphorylated pBR322. Plasmids containing $K2_{HPg}K3_{HPg}$ cDNA were identified by digestion with *Bam*HI. Both strands of the insert were sequenced.

The $K2_{HPg}K3_{HPg}$ insert was ligated into the *Bam*HI restriction site of pQE-8 and transformed into the strain M15 containing the repressor plasmid pREP4. The direction of the insert was determined in different clones by digestion with *Nco*I (Figure 2).

Expression and Isolation. The expression and isolation of r-$K2_{HPg}K3_{HPg}$ were carried out according to the method of Marti *et al.* (1994). The cells were grown at 37 °C in $2\times YT_{amp/kana}$ medium in 2 L round-bottomed flasks to an A_{600} of about 0.7−0.9. IPTG was added to a final concentration of 1 mM to induce the production of the r-$K2_{HPg}K3_{HPg}$. The cells were grown for another 4.5 h at 37 °C and harvested by centrifugation for 30 min (4000*g*, 4 °C). The cell paste was stored at −80 °C.

To verify the production of r-$K2_{HPg}K3_{HPg}$, the cell proteins were separated on 15 % SDS/polyacrylamide gels and blotted on nitrocellulose membranes. Novel bands were detected by Ponceau S staining and by an ELISA based on polyclonal antibodies against native HPg.

To isolate r-$K2_{HPg}K3_{HPg}$, the thawed cell paste was suspended in extraction buffer (6 M guanidine hydrochloride in 0.1 M sodium phosphate, pH 8) (5 mL/g of cell paste). The suspension was stirred for 1 h at room temperature and centrifuged for 30 min (15000*g*, 4 °C). β-Mercaptoethanol was added to the supernatant and all extraction buffers to 10 mM final concentration. The supernatant was loaded on a Ni^{2+}−NTA/agarose column (1.5 cm × 5 cm) equilibrated against extraction buffer, pH 8. The column was washed successively with extraction buffer, pH 8, and then pH 6.3. The r-$K2_{HPg}K3_{HPg}$ was eluted with extraction buffer, pH 5.

Refolding and Purification. The refolding of r-$K2_{HPg}K3_{HPg}$ was carried out following a protocol similar to that of Cleary *et al.* (1989). The pH of the effluent was adjusted to 8, and DTT was added to a 5 mM concentration. After being stirred overnight, the solution was diluted with 4 vol of 50 mM Tris/HCl, pH 8, containing 1.25 mM each of reduced and oxidized glutathione, with 1 h intervals between each addition, and stirred for 6 h at 4 °C. The renatured protein was dialyzed against water for 2 days and for an additional 2 days against 50 mM sodium phosphate buffer, pH 8 (loading buffer). The dialysate was loaded on a lysine-Bio-Gel column (2 cm × 13 cm) equilibrated against loading buffer and the column was washed with the same buffer. Adsorbed r-$K2_{HPg}K3_{HPg}$ was eluted with loading buffer containing 50 mM 6-AHA. The r-$K2_{HPg}K3_{HPg}$ containing pool was lyophilized and desalted on a Sephadex G-50 f column (1.5 cm × 100 cm) equilibrated against 50 mM ammonium hydrogen carbonate and lyophilized.

RP-HPLC. RP-HPLC was carried out on an Aquapore butyl column (2.1 mm × 100 mm, wide pore 30 nm, 7 μm, Applied Biosystems). A Hewlett Packard liquid chromatograph was used with acetonitrile gradients (Figure 3).

Enzymatic Cleavage. r-$K2_{HPg}K3_{HPg}$ was incubated with FXa, isolated and activated as described by Nagai and Thøgersen (1987), in 50 mM Tris/HCl, pH 8, containing 100 mM sodium chloride, for 24 h at 37 °C, at an enzyme/substrate ratio of 1:100, as reported by the same authors.

Preparation of Individual Recombinant Kringle Modules. r-$K2_{HPg}$ (r-C162T/E163S/EE [$K2_{HPg}$/C169G]T) and r-$K3_{HPg}$ (r-TYQ[$K3_{HPg}$]DS; Cys^{297}-glutathione) were cloned, expressed, and folded as published (Marti *et al.*, 1994).

Preparation and Proteolytic Fragmentation of Plasminogen. Glu−HPg was purified from outdated blood plasma (Pittsburgh Central Blood Bank, Pittsburgh, PA) via affinity chromatography on L-lysine-Sepharose (Deutsch & Mertz, 1970). $K1_{HPg}K2_{HPg}K3_{HPg}$ (segment Tyr^{80}−Val^{338} of HPg) $K4_{HPg}$ (segment Val^{355}−Ala^{440} of HPg), and miniplasminogen ($K5_{HPg}$+protease, segment Ser^{441}−Asn^{791} of Hpg) fragments were prepared by elastase digestion of HPg and purified also by affinity chromatography on L-lysine-Sepharose (Sottrup-Jensen *et al.*, 1978). $K1_{HPg}$ (segment Tyr^{80}−Glu^{165} of HPg)

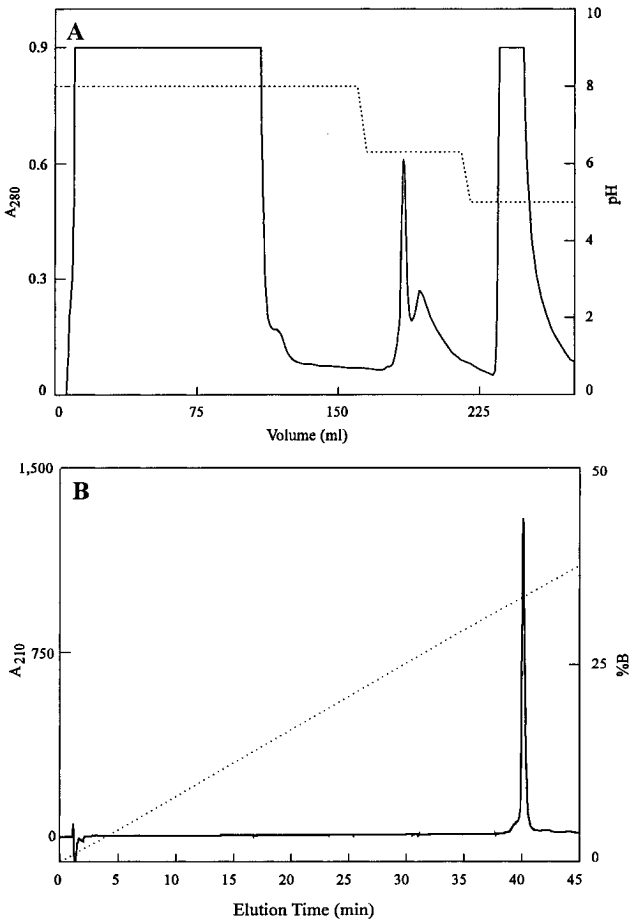

FIGURE 3: (A) Isolation of r-$K2_{HPg}K3_{HPg}$ by Ni chelate affinity chromatography. The supernatant of 18 g of induced *E. coli* cells M15 was loaded on a Ni^{2+}−NTA/agarose column (1.5 cm × 5 cm) equilibrated against extraction buffer, pH 8, at a flow rate of 0.5 mL/min. The column was successively washed with extraction buffer, pH 8.0 and 6.3. r-$K2_{HPg}K3_{HPg}$ was eluted at pH 5.0. To the supernatant and all extraction buffers β-mercaptoethanol was added to a 10 mM concentration. (B) RP-HPLC analysis of r-$K2_{HPg}K3_{HPg}$. r-$K2_{HPg}K3_{HPg}$ eluted from lysine-Bio-Gel was analyzed on a Aquapore Butyl column (2.1 mm × 100 mm, wide pore, 30 nm, 7 μm) using a linear acetonitrile gradient (0%−50 % solution B in 60 min). Solution A: 0.1% (by volume) TFA in distilled water (HPLC grade). Solution B: 0.1% (by volume) TFA and 80% (by volume) acetonitrile in distilled water (HPLC grade). Flow rate, 0.3 mL/min.

was obtained by digestion of the $K1_{HPg}K2_{HPg}K3_{HPg}$ with *Staphylococcus aureus V8* protease and purified as published (Motta *et al.*, 1986).

Detection of Free Thiol Groups. r-$K2_{HPg}K3_{HPg}$ was dissolved in 50 mM Tris/HCl, pH 8, iodoacetamide was added in a 10-fold molar excess, and the mixture was incubated for 1 h in the dark. The reaction was stopped by adjusting the pH to 2. Desalting was carried out on a Sephadex G-15 column (1.8 cm × 20 cm) equilibrated against 0.13 M formic acid.

Amino Acid Analysis. Samples were hydrolyzed in the gas-phase with 6 M hydrochloric acid containing 0.1 % (v/v) phenol for 24 h at 115 °C under vacuum according to Chang and Knecht (1991). The liberated amino acids were reacted with phenylisothiocyanate and the resulting phenylthiocarbamyl amino acids analyzed by RP-HPLC on a Nova Pak C18 column (3.9 mm × 150 mm, 4 μm; Waters) in a Hewlett Packard liquid chromatograph according to Bidlingmeyer *et al.* (1984). The 0.14 M sodium acetate buffer, pH 6.4, was replaced by the corresponding ammonium acetate buffer.

Amino Acid Sequence Analysis. N-terminal amino acid sequence analysis was carried out using Edman degradation in a pulsed liquid-phase sequenator 477A from Applied Biosystems using a program adapted from Hunkapiller *et al.* (1983). The released amino acids were analyzed on-line.

Molecular Mass Analysis. The mass of the r-$K2_{HPg}K3_{HPg}$ was determined by using electrospray mass spectrometry (VG Platform; Fisons Instruments).

^1H NMR Spectroscopy. For the NMR analyses, the protein samples were pre-exchanged against ^2H_2O, lyophilized, and dissolved into 0.35 mL of ^2H_2O (99.996 atom % ^2H, Isotec Inc., OH). Final protein concentrations were about 0.6 mM. The pH* was adjusted by additions of dilute ^2HOAc or NaO^2H. Probe temperature was calibrated with an ethylene glycol standard. Chemical shifts are by reference to the sodium 3-(trimethylsilyl)-(2,2,3,3-$^2H_4$) propionate signal, using p-dioxane as an internal standard (De Marco, 1977). ^1H NMR spectra were recorded at 500 MHz on a Bruker AM-500 spectrometer equipped with an Aspect 3000 mini-computer. The residual solvent $^1H^2HO$ signal was suppressed by gated low-power irradiation during the relaxation delay of 1.2−2.0 s introduced between scans. Data were collected in quadrature detection mode with a spectral width of 6500 Hz. ^1H NMR COSY (Jeener *et al.*, 1979; Marion & Wüthrich, 1983), NOESY (Kumar *et al.*, 1980), and TOCSY (Bax & Davis, 1985) spectra were recorded in the phase-sensitive mode using time-proportional phase incrementation. For each experiment, 512 t_1 increments of 2K complex t_2 points were acquired. ^1H NMR ligand (6-AHA) titration experiments were performed by adding measured aliquots of a concentrated ligand solution to the protein samples and the data were analyzed as reported (De Marco *et al.*, 1987). The program FELIX, version 2.3 (BIOSYM, California), was used for the NMR data processing.

RESULTS

Recombinant Kringle (2 + 3) Expression. The cDNA construct EE[$K2_{HPg}K3_{HPg}$]DS corresponding to the Glu^{164}−Ser^{335} sequence of Pg was cloned into the protein expression plasmid pQE-8 and expressed in the *E. coli* strain M15. A preliminary identification of the recombinant protein by Western blotting indicated a relatively high expression level. The SDS/PAGE and the immunoblot showed a main band at the expected molecular mass of 21.6 kDa for r-$K2_{HPg}K3_{HPg}$ (not shown). After 4.5 h of protein expression, 3.3 g of wet cells/L of medium was collected. The r-$K2_{HPg}K3_{HPg}$ was obtained by Ni^{2+}−NTA/agarose affinity chromatography (Figure 3A) with a yield of 1.2 mg/g wet cells.

The refolding of r-$K2_{HPg}K3_{HPg}$ was monitored by RP-HPLC on an Aquapore Butyl column. The chromatogram was characterized by a broad, heterogeneous elution zone for the crude material which evolved into a prominent, rather symmetrical peak for the folded protein. The final purification was achieved by affinity chromatography on lysine-Bio-Gel. The purified material eluted as a sharp, symmetrical peak upon RP-HPLC on an Aquapore butyl column (Figure 3B).

The treatment of r-$K2_{HPg}K3_{HPg}$ with FXa was only partially successful and was therefore abandoned since elimination

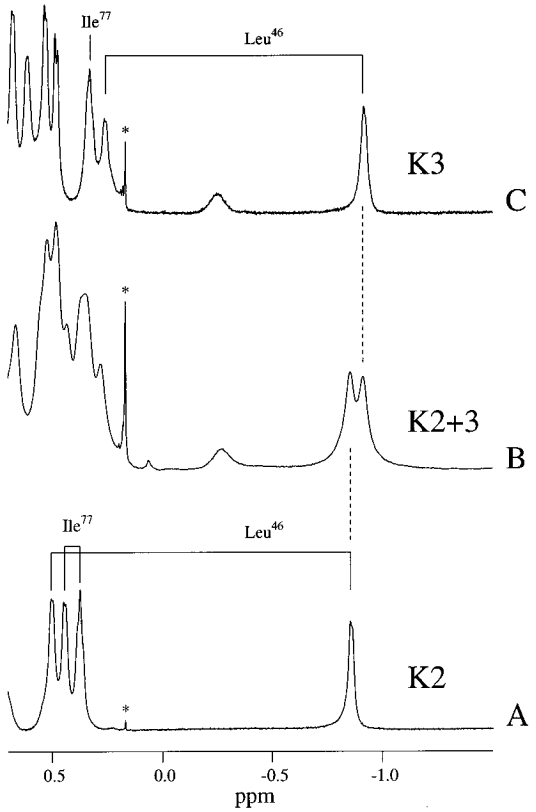

FIGURE 4: 500 MHz ^{1}H NMR spectra of plasminogen r-K2$_{HPg}$ (A), r-K2$_{HPg}$K3$_{HPg}$ (B), and r-K3$_{HPg}$ (C): high-field-shifted methyl resonances. Leu^{46} $CH_3^{\delta,\delta'}$, Ile^{77} CH_3^{γ} (doublet), and CH_3^{δ} (triplet) signals are labeled in spectra A and C. The corresponding signals can be recognized, by comparison, in the spectrum of r-K2$_{HPg}$K3$_{HPg}$. Leu^{46} CH_3^{δ} to $CH_3^{\delta'}$ scalar connectivities were established via COSY and TOCSY experiments. An asterisk denotes an impurity signal. Protein concentrations are about 0.6 mM, dissolved in D_2O (^2H_2O), pH* 5.0, 310 K.

of the hexahistidine tag sequence was incomplete and, in some of the material, a secondary cleavage site occurred within the K3$_{HPg}$ sequence. Consequently, the chemical characterization and ^{1}H NMR analyses were carried out on protein bearing the attached N-terminal fusion tail.

Automated Edman degradation of r-K2$_{HPg}$K3$_{HPg}$ yielded the expected (Figure 2) N-terminal sequence: M-R-G-S-H-H-H-H-H-H-G-S-I-E-G-R-E-E-. The amino acid composition yielded values compatible with the reported sequence (Figure 1). The molecular mass of 21 567.9, determined by mass spectrometry, agrees closely with 21 564.8, the value calculated from the primary structure. The combined data thus indicates that the sequence was correctly translated down to the stop codon following Ser^{335}.

The absence of CM-cysteine precludes the existence of unpaired half-cystines with free −SH in the expressed protein. Likewise, disulfide bridge formation of kringle half-cystines with other partners, such as glutathione of the refolding medium can be excluded both from compositional as well as from mass spectrometric data.

NMR Analysis. ^{1}H NMR spectra of the homologous domains K1$_{HPg}$, K4$_{HPg}$, and K5$_{HPg}$ (Llinás *et al.*, 1983; Thewes *et al.*, 1987), K2$_{tPA}$ (Byeon *et al.*, 1989), and K$_{uPA}$ (Bogusky *et al.*, 1989; Bokman *et al.*, 1993) are similar in that they all exhibit a characteristic, shifted CH_3^{δ} doublet arising from the conserved Leu^{46} residue. Its consistent high-field position at ∼−1.0 ppm originates from anisotropic ring current effects stemming from neighboring aromatic side

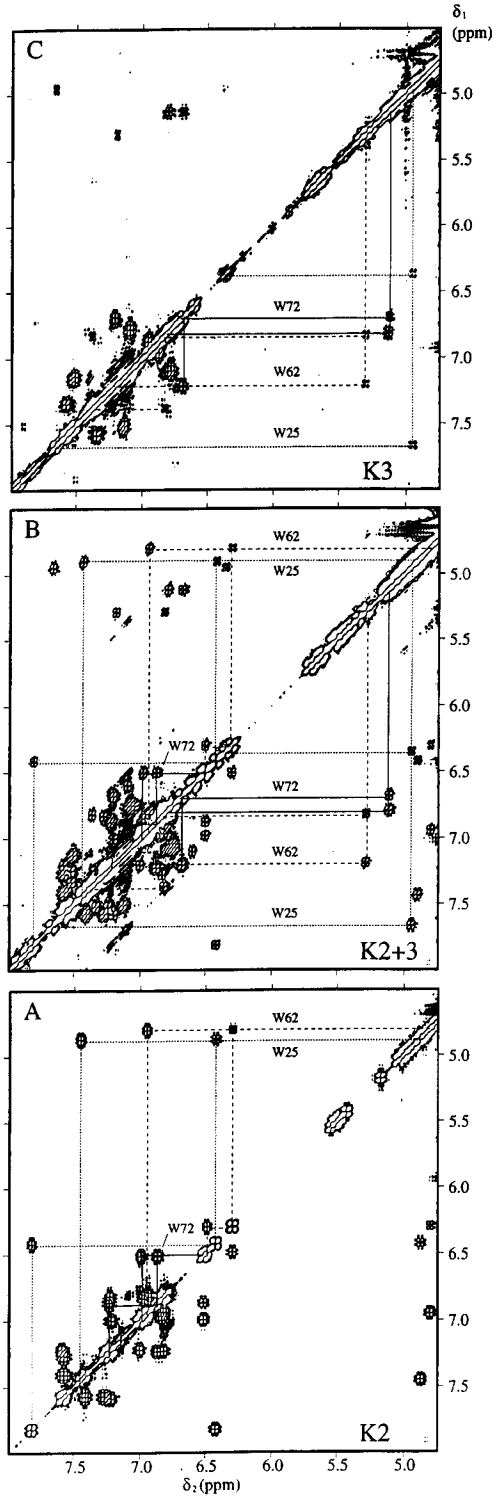

FIGURE 5: ^{1}H NMR 2D COSY spectra of r-K2$_{HPg}$ (A), r-K2$_{HPg}$-K3$_{HPg}$ (B), and r-K3$_{HPg}$ (C): aromatic regions. Trp^{25} (W25), Trp^{62} (W62), and Trp^{72} (W72) indole ring connectivities for K2 and K3 are indicated within the upper left and lower right quadrants, respectively. Experimental conditions as for Figure 4.

chains within the modules' hydrophobic core (Ramesh *et al.*, 1987). Jointly with the strictly conserved Trp^{25} and Trp^{62} residues, Leu^{46} nucleates a hydrophobic core subjacent to the canonical "lysine binding site" (Llinás *et al.*, 1985; Ramesh *et al.*, 1987). Expansions of high-field ^{1}H NMR spectra of r-K2$_{HPg}$, r-K2$_{HPg}$K3$_{HPg}$, and r-K3$_{HPg}$ are shown in Figure 4. Inspection of the latter enables one to readily identify the conserved Leu^{46} δ-methyl doublets at −0.87 (r-K2$_{HPg}$) and −0.94 ppm (r-K3$_{HPg}$) as well as the Ile^{77} γ-

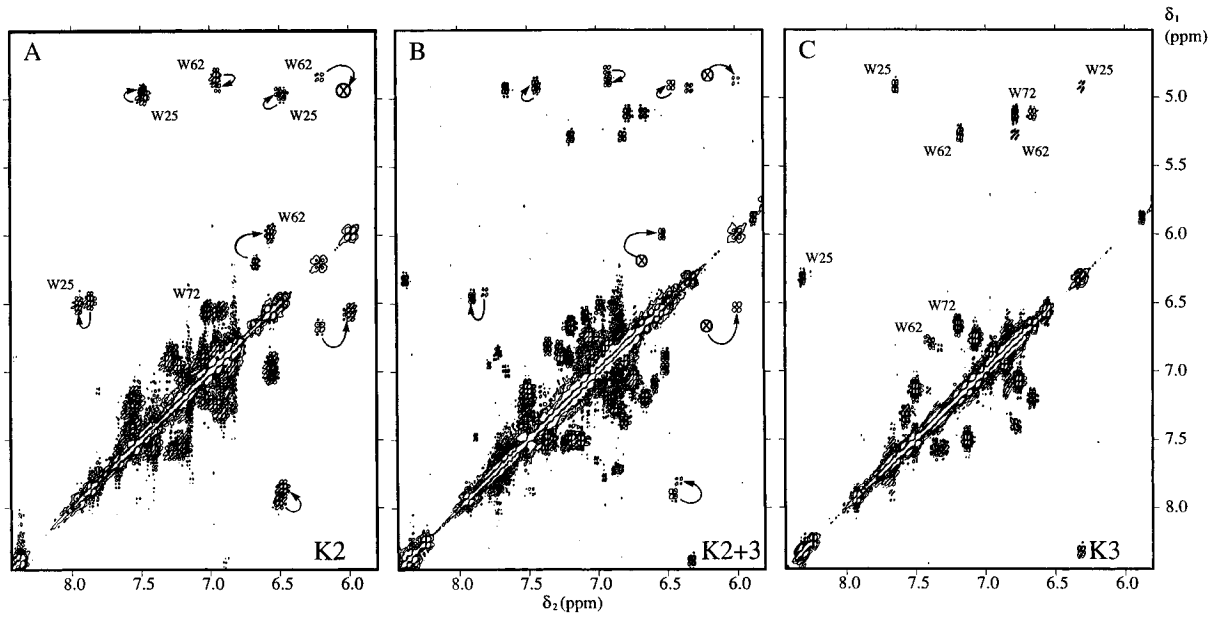

FIGURE 6: ^{1}H NMR 2D COSY spectra of r-K2$_{HPg}$ (A), r-K2$_{HPg}$K3$_{HPg}$ (B), and r-K3$_{HPg}$ (C): ligand binding effects on Trp signals. Each panel depicts the superposition of two spectra, one recorded in the absence of ligand and the other recorded in the presence of a 5-fold excess of 6-AHA. Arrows indicate ligand-induced shifts: they arise from r-K2$_{HPg}$ signals, r-K3$_{HPg}$ being unaffected by ligand. Cross-peaks detected in lower-contour plots are denoted by ⊗. Spectra recorded at pH* 7.2; other experimental conditions as for Figure 4.

and δ-methyl signals as they exhibit essentially identical chemical shifts in all three spectra, an indication that the folding of the individual K2$_{HPg}$ and K3$_{HPg}$ domains is preserved in the intact r-K2$_{HPg}$K3$_{HPg}$ construct.

In lysine-binding kringles, aromatic residues contribute an important lipophilic component to the exposed binding site surface (Llinás *et al.*, 1985; Tulinsky *et al.*, 1988b). Figure 5 depicts aromatic region expansions of 2D COSY spectra from r-K2$_{HPg}$, r-K2$_{HPg}$K3$_{HPg}$, and r-K3$_{HPg}$. As described above for the aliphatic high-field spectra (Figure 4), the r-K2$_{HPg}$K3$_{HPg}$ aromatic spectrum (Figure 5B) reveals itself essentially as a superposition of spectra stemming from the individual r-K2$_{HPg}$ (A) and r-K3$_{HPg}$ (C) modules, implying that the overall foldings of K2 and K3 within the intact K2$_{HPg}$-K3$_{HPg}$ are, *prima facie*, little affected by the interdomain Cys^{169}−Cys^{297} bridge. Spin system identifications, based on COSY and TOCSY experiments and comparison of spin−spin connectivity patterns in spectra from homologs, have led to the spectral assignments while supporting a folding relatedness to the previously investigated kringles. Furthermore, NOESY spectra (not shown) reveal that the Leu^{46} δ-methyl group in r-K2$_{HPg}$, r-K3$_{HPg}$, and r-K2$_{HPg}$K3$_{HPg}$ are located next to the indole rings of Trp^{25} in the domains' hydrophobic cores, as formerly described for the homologs [see, e.g., Ramesh *et al.* (1987)].

Tryptophan fluorescence quenching experiments and affinity chromatography on lysine-Bio-Gel have indicated that 6-AHA interacts with r-K2$_{HPg}$ (Marti *et al.*, 1994). Figure 6 shows 6-AHA binding effects on the aromatic signals of the r-K2$_{HPg}$ (A), r-K2$_{HPg}$K3$_{HPg}$ (B), and r-K3$_{HPg}$ (C), where each panel depicts the superposition of spectra recorded in the absence and in the presence of a 5-fold molar excess of 6-AHA. It is apparent that while K2$_{HPg}$ resonances undergo ligand-induced shifts (panel A *vs* B), the K3$_{HPg}$ resonances remain unperturbed (panel B *vs* C). Hence, it follows that 6-AHA acts selectively, as a ligand that interacts with the K2$_{HPg}$ binding site but not with the corresponding locus in K3$_{HPg}$. Analysis of ^{1}H NMR ligand titration data for r-K2$_{HPg}$ (not shown) and r-K2$_{HPg}$K3$_{HPg}$ (Figure 7) enables values for

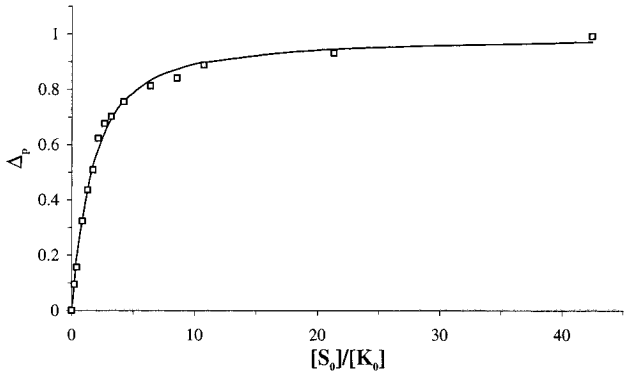

FIGURE 7: Titration of r-K2$_{HPg}$K3$_{HPg}$ with 6-AHA. The fraction of ligand-bound kringle (Δ_p) is shown as a function of the ratio of total ligand to total kringle concentration ([S$_o$]/[K$_o$]). Experiments were performed at pH*7.2, 298 K.

the equilibrium association constant (K_a) to be derived (De Marco *et al.*, 1987). One thus obtains $K_a \approx 2.3 \pm 0.2$ mM^{-1} and 2.2 ± 0.3 mM^{-1} for r-K2$_{HPg}$ and r-K2$_{HPg}$K3$_{HPg}$, respectively, which indicates that the affinity of K2$_{HPg}$ for 6-AHA is essentially unaffected by the disulfide-linked K3$_{HPg}$ domain in r-K2$_{HPg}$K3$_{HPg}$. It is gratifying that the K_a's for 6-AHA determined by NMR closely agree with the value 2.5 mM^{-1} previously estimated from the fluorescence quenching experiments (Marti *et al.*, 1994).

The Leu^{46} δ-methyl signals in r-K2$_{HPg}$ and r-K3$_{HPg}$, at −0.85 and −0.93 ppm, respectively (same chemical shifts for the individual components in r-K2$_{HPg}$K3$_{HPg}$), appear somewhat low-field shifted relative to those observed for the Leu^{46} methyl group in K1$_{HPg}$, K4$_{HPg}$, and K5$_{HPg}$ (∼−1.0 ppm; Thewes *et al.*, 1987). A lingering doubt is whether the Leu^{46} environments in r-K2$_{HPg}$K3$_{HPg}$ are the same as for the same residues within the corresponding domains in intact HPg. To address this question we have recorded spectra of the K1$_{HPg}$K2$_{HPg}$K3$_{HPg}$ fragment and of intact Glu−HPg. Figure 8 (traces A−D) illustrates high-field methyl resonances of Glu−HPg ($M_r \approx 93$ kDa), fragment K1$_{HPg}$K2$_{HPg}$-K3$_{HPg}$ ($M_r \approx 30$ kDa), r-K2$_{HPg}$K3$_{HPg}$ ($M_r \approx 21$ kDa), and

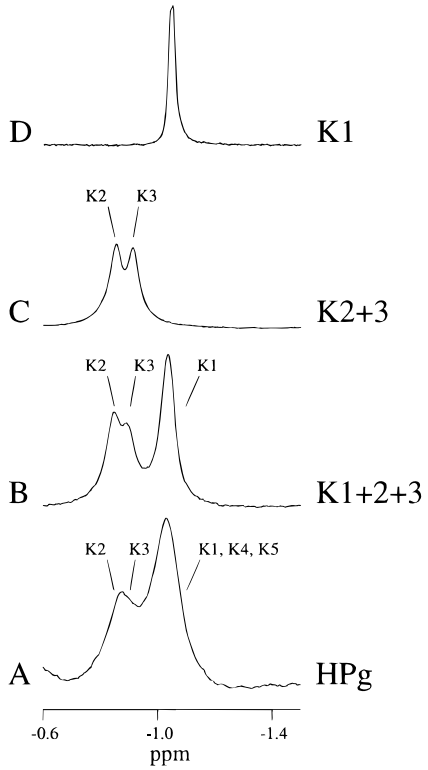

FIGURE 8: ^{1}H NMR spectra of intact Glu-plasminogen (A), $K1_{HPg}$-$K2_{HPg}K3_{HPg}$ fragment (B), r-$K2_{HPg}K3_{HPg}$ (C), and $K1_{HPg}$ (D): high-field Leu^{46} CH_3^{δ} resonances. $K4_{HPg}$ and $K5_{HPg}$ (not shown) exhibit similar Leu^{46} CH_3^{δ} resonance as $K1_{HPg}$ (Thewes *et al.*, 1987). It is apparent that the $K2_{HPg}K3_{HPg}$ Leu^{46} CH_3^{δ} signals are present in spectra of $K1_{HPg}K2_{HPg}K3_{HPg}$ and intact plasminogen at about the same chemical shifts. Experimental conditions are as for Figure 6.

$K1_{HPg}$ ($M_r \approx 10$ kDa). Chemical shifts of Leu^{46} δ -methyl signals are -1.04, -0.87, -0.94, -1.05, and -1.04 ppm for the isolated $K1_{HPg}$ (Figure 8D), r-$K2_{HPg}$ (Figure 4A), r-$K3_{HPg}$ (Figure 4C), $K4_{HPg}$ (Llinás *et al.*, 1983), and $K5_{HPg}$ (Thewes *et al.*, 1987), respectively. Despite obvious line width differences reflecting the relative molecular mass of the various constructs, the Leu^{46} signals arising from individual kringles are essentially invariant from one spectrum to the other. Thus, it is implied that the individual modules obtained via either proteolytic fragmentation of intact HPg or recombinant gene expression possess foldings essentially identical to those they exhibit in the native, intact protein. The above evidence also assigns the five high-field Leu^{46} δ-methyl signals apparent in a ^{1}H NMR spectrum of HPg previously reported by Teuten *et al.* (1991).

DISCUSSION

Kringles 2 and 3 of human plasminogen were expressed and refolded as disulfide-linked tandem domains. Its isolation and purification from the cell lysate was greatly facilitated by using affinity chromatography on Ni^{2+}−NTA/agarose and on lysine-Bio-Gel. The final product chromatographed as a sharp, single peak when analyzed by RP-HPLC (Figure 3B).

The expression level of r-$K2_{HPg}K3_{HPg}$ of 1.2 mg/g of wet cells was comparable to that of r-$K3_{HPg}$ [1.7 mg/g, Marti *et al.* (1994)]. To ensure optimal yields of the protein it was essential to maintain reducing conditions in the elution buffer of the Ni^{2+}−NTA/agarose column and to allow sufficient time for the refolding process. The protein analytical data indicated that the expressed sequence was correct and that translation terminated at the introduced stop codon.

The final processing of the expressed protein, *i.e.*, the removal of the hexahistidine tag by FXa, was hampered by incomplete scission of the FXa cleavage site at the N-terminus of K2 and by the appearance of a secondary cleavage site at Arg^{265}−Gly^{266} in the N-terminal region of the K3 sequence. This latter cleavage has been observed and identified in previous investigations of r-$K3_{HPg}$ (Marti *et al.*, 1994). To avoid sample heterogeneity, the FXa treatment was omitted and all subsequent investigations were performed on a protein carrying the N-terminal fusion tail.

As illustrated in Figure 1, r-$K2_{HPg}K3_{HPg}$ contains two types of cystine bridges: six intrakringle S−S bonds, arranged in a characteristic 1−6, 2−4, 3−5 pattern within each domain, and an interkringle link between Cys^{169} and Cys^{297}. The half-cystine pairing of the intrakringle bridges was primarily corroborated by the ^{1}H NMR spectra which exhibited the typical features of the correctly folded domains. Additional proof of correct folding and half-cystine pairing is provided, at least for the K2 domain within the construct, by its lysine affinity (Marti *el al.*, 1994), whose manifestation depends directly on a native three-dimensional structure.

Formation of the interkringle disulfide bond between Cys^4 of K2 and Cys^{43} of K3 was verified indirectly by mass spectrometry and determination of the amino acid composition. Data obtained with both methods exclude a reaction of the free −SH groups with glutathione of the refolding medium or with iodoacetamide in the alkylation experiment. Since the mass spectrum revealed only the mass of the monomeric r-$K2_{HPg}K3_{HPg}$, formation of polymers via intermolecular disulfide bonds can also be excluded. From the combined evidence it may hence be concluded that the half-cystines at positions 169 and 297 are indeed paired to yield the proper interkringle disulfide bridge.

$K1_{HPg}$, $K4_{HPg}$, and $K5_{HPg}$ exhibit definite binding affinity for L-lysine ($K_a \approx 3$, 24, and 0.1 mM^{-1}, respectively) and other zwitterionic ω-amino acid analogs such as 6-AHA ($K_a \approx 60$, 22, and 11 mM^{-1}, respectively) (De Marco *et al.*, 1987; Ramesh *et al.*, 1987; Thewes *et al.*, 1990; Rejante, 1992). By comparison, the affinities toward 6-AHA we have determined for K2 in r-$K2_{HPg}$ and in r-$K2_{HPg}K3_{HPg}$ (Figure 5) are relatively weak ($K_a \approx 2.3 \pm 0.2$ mM^{-1}), while the covalently attached $K3_{HPg}$ domain shows no measurable interaction with the ligand (Figure 6C). Thus, as proposed earlier (Marti *et al.*, 1994), the different Pg kringles rank $K1_{HPg} > K4_{HPg} > K5_{HPg} > K2_{HPg} > K3_{HPg}$ vis-à-vis the strength of their interactions with 6-AHA. The negligible interaction of 6-AHA with $K3_{HPg}$ was predictable considering that $K3_{HPg}$ lacks the Asp^{57} residue that is crucial for ion-pairing the cationic end of the ligand molecule (Trexler *et al.*, 1982; Byeon *et al.*, 1995).

The Leu^{46} δ-methyl signals of r-$K2_{HPg}$ (-0.87 ppm) and r-$K3_{HPg}$ (-0.94 ppm) (Figure 4) are low-field shifted relative to the corresponding signals in $K1_{HPg}$ (-1.04 ppm), $K4_{HPg}$ (-1.05 ppm), and $K5_{HPg}$ (-1.04 ppm). The lesser shielding of the Leu^{46} δ-methyl protons in $K2_{HPg}$ and $K3_{HPg}$ reflect an altered array of the aromatic rings that contact the Leu^{46} side chain within the hydrophobic core which structurally supports the binding site. On the other hand, kringles exhibit rather unique Trp aromatic connectivities, with resonances characteristically well dispersed within an approximate $8.5 > \delta > 4.5$ ppm range (Ramesh *et al.*, 1986; Motta *et al.*, 1987;

Byeon *et al.*, 1989; Thewes *et al.*, 1990; Bokman *et al.*, 1993) which contrast the Trp^{72} indole signals in $K2_{HPg}$ that appear closer to those in unstructured, "random coil" polypeptides (Figure 5A). This suggests that by comparison to what is the case in other kringles, in r-$K2_{HPg}$ the Trp^{72} indole ring is structurally less constrained on the protein surface. Thus, although the $K2_{HPg}$ and $K4_{HPg}$ have highly conserved amino acid residues neighboring the ligand binding site, the Trp^{72} aromatic spectrum reflects a subtle structural variability that may relate to the 10-fold difference in their affinities for 6-AHA binding as it is now well established that Trp^{72} is directly involved in ligand−kringle interactions (Hochschwender & Laursen, 1981; Llinás *et al.*, 1983).

Finally, in the kringle (1 + 2 + 3) ^1H NMR spectrum, the subset of kringle (2 + 3) signals exhibits resonances that are considerably broader than those stemming from the K1 component (see, *e.g.*, Figure 8B). Such evidence strongly suggests that the mobility of the individual domains within the kringle (2 + 3) unit is considerably reduced, relative to that of the attached K1 module. This may be attributed to the extra constraint imposed by the Cys^{169}−Cys^{297} bridge on the kringle (2 + 3) dynamics since both r-$K2_{HPg}$ and r-$K3_{HPg}$ exhibit resonances which are narrower than those apparent in the spectrum of r-$K2_{HPg}K3_{HPg}$ (Figure 4). Thus, the *ansatz* that the kringle (2 + 3) construct should be considered a novel supermodule becomes strengthened. The functional significance of this evolutionary conserved structure remains to be elucidated.

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